Post-natal maturation of the hyperpolarisation-activated cation current, \( I_h \), in trigeminal sensory neurons

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Abstract

Hyperpolarisation-activated inward currents (I\textsubscript{h}) contribute to neuronal excitability in sensory neurons. Four subtypes of hyperpolarisation-activated, cyclic nucleotide-gated (HCN) channels generate I\textsubscript{h}, with different activation kinetics and cAMP sensitivities. The aim of the present study was to examine the postnatal development of I\textsubscript{h} and HCN channel subunits in trigeminal ganglion (TG) neurons. I\textsubscript{h} was investigated in acutely dissociated TG neurons from rats aged between 1 to 35 postnatal (P) days using whole-cell patch-clamp electrophysiology. In voltage-clamp studies, I\textsubscript{h} was activated by a series of hyperpolarising voltage steps from -40 mV to -120 mV in -10 mV increments. Tail currents from a common voltage step (-100 mV), were used to determine I\textsubscript{h} voltage dependence. I\textsubscript{h} activation was faster in older rats and occurred at more depolarised potentials; the half maximal activation voltage (V\textsubscript{1/2}) changed from -89.4 mV (P1) to -81.6 mV (P35). In current-clamp studies, blocking I\textsubscript{h} with ZD7288 caused membrane hyperpolarisation and increases in the action potential half-duration at all postnatal ages examined. ZD7288 also reduced the action potential firing frequency in multiple firing neurons. Western blot analysis of the trigeminal ganglion detected immunoreactive bands corresponding to all HCN subtypes. The HCN1 and HCN2 band density increased with postnatal age whereas the low intensity HCN3 and moderate intensity HCN4 bands were not changed. This study suggests that functional I\textsubscript{h} currents are activated in rat trigeminal sensory neurons from P1 during postnatal development, have an increasing role with age, and modify neuronal excitability.

Key words

HCN, I\textsubscript{h}, postnatal development, trigeminal ganglion, ZD7288
**Abbreviations** ZD7288, 4-((N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino)
pyrimidinium chloride, TG, trigeminal ganglia, DRG, dorsal root ganglia, HCN, hyperpolarisation-activated, cyclic nucleotide-gated channels, I_h, hyperpolarisation activated inward current, RT, room temperature.
Introduction

The processing and modulation of somatosensory information changes dramatically following birth in both rodent and human. This postnatal maturation of the sensory system is due to changes in the neurochemical phenotype of primary afferent neuronal soma and terminals, myelination of axons, maturation of central synaptic connections with second order neurons, and the development of interneuron circuits and descending inhibitory inputs (Fitzgerald 2005). The expression of ion channels in primary afferent neurons changes considerably during the first three postnatal weeks in rats, reaching a mature state after this time (Fitzgerald and MacDermott 2005). The excitability of sensory ganglion neurons is determined by ion channels properties and changes with postnatal age (Fitzgerald and Fulton 1992).

One family of ion channels that can strongly influence neuronal excitability, the hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels, occur in many excitable cells including neurons, cardiac pacemaker cells, and photoreceptors (for reviews (Pape 1996; Robinson and Siegelbaum 2003)). In adult sensory neurons, the current of HCN channels, $I_h$, contributes to neuronal excitability. The persistent activation of $I_h$ at resting and more negative membrane potentials counterbalances hyperpolarisation and brings the membrane back towards action potential threshold following an afterhyperpolarisation (AHP) (Hogan and Poroli 2008; Masuda et al. 2006; Momin et al. 2008; Orio et al. 2009).

Four HCN channel subtypes have been cloned (Ludwig et al. 1998; Santoro et al. 1998) and all subtypes are detected in the trigeminal ganglion (TG) (Cho et al. 2009a; Cho et al. 2009b; Wells...
et al. 2007) and dorsal root ganglion (DRG) (Antal et al. 2004; Chaplan et al. 2003; Cho et al. 2009b; Jiang et al. 2008; Kouranova et al. 2008; Matsuyoshi et al. 2006; Moosmang et al. 2001; Tu et al. 2004), although the different reports are not entirely consistent. Most of these studies show that HCN protein is localised to neuronal membranes. When expressed individually, each subtype shows different activation kinetics, voltage dependence and sensitivity to cAMP (Moosmang et al. 2001; Stieber et al. 2005). HCN1 has the fastest opening kinetics followed by HCN2, HCN3 and HCN4. HCN2 and HCN4 are highly sensitive to cAMP and their activation voltage can be shifted by more than 20 mV in a depolarising direction with elevated cAMP. HCN1 and HCN3 are less sensitive to cAMP but are activated at more depolarised potentials than HCN2 and HCN4. In addition, HCN channels can be assembled as either homomeric or heteromeric tetramers and have mixed characteristics (Chen et al. 2001; Much et al. 2003).

Postnatal changes in HCN channel protein expression and I_h have been observed in the central nervous system (Bayliss et al. 1994; Bender et al. 2001; McCormick and Prince 1987; Surges et al. 2006), cardiac neurons (Adams et al. 2002; Hogg et al. 2001) and mesencephalic trigeminal neurons (Tanaka et al. 2003). However, it is not clear whether I_h is developmentally regulated in the major somatosensory ganglion (DRG or TG) neurons or whether there are functional consequences of such regulation.

The aim of this study was to determine whether I_h or HCN channel subunit expression changes during postnatal development and how such regulation affects the excitability of trigeminal ganglion neurons.
Methods

Ethical approval

Experiments were conducted on Sprague-Dawley rats of 1 to 35 postnatal days (P) from the Anatomy and Cell Biology colony at the University of Melbourne. All procedures conformed to the Australian National Health and Medical Research Council code of practice for the use of animals in research and were approved by the University of Melbourne Animal Experimentation Ethics Committee. They also comply with the guidelines of the Committee for Research and Ethical Issues of IASP (Zimmermann 1983).

Acute dissociation of trigeminal ganglion

Sprague-Dawley rats were deeply anaesthetised with isoflurane (4%) and decapitated. Both trigeminal ganglia were rapidly removed, placed in ice-cold, oxygenated dissociation solution, consisting of (in mM) 140 NaCl, 5 KCl, 10 HEPES, 0.5 CaCl$_2$, 2 MgCl$_2$ and 10 D-glucose (pH = 7.3) and cut into 3 to 4 pieces with iridectomy scissors. The tissues were then incubated for 25 min at 37 °C in dissociation solution containing 0.6 to 1.2 mg ml$^{-1}$ of collagenase (Type1A, Sigma). After washing tissues, individual neurons were dissociated by gentle trituration using sterilized Pasteur pipettes with decreasing bore and fire-polished tips in Dulbecco’s Modified Eagle’s Medium (Sigma). Cells were placed onto 35 mm plastic tissue culture dishes and allowed to settle down for more than 2 hours in 5% CO$_2$/air incubator at 37 °C. Experiments were conducted from short-term cultures (2 to 7 hours after plating) to minimize alterations in cellular phenotype (Schlichter et al. 1991).
**Electrophysiology**

After incubation, cells were perfused with oxygenated (100% O₂) HEPES buffered saline, consisting of (in mM) 140 NaCl, 5 KCl, 10 HEPES, 2 CaCl₂, 2 MgCl₂ and 10 D-glucose (pH = 7.3, 300 mOsmol l⁻¹) at approximately 1.0 ml min⁻¹. Experiments were carried out at room temperature to enable comparison to previous studies and at 33 to 35 °C because Iₜ is highly temperature sensitive (Q₁₀ = 3 to 5; Hogg et al. 2001; Magee 1998; Orio et al. 2009; Pape 1996). Standard whole-cell patch-clamp electrophysiology was done with an Axopatch 200B amplifier, Digidata 1200 analogue-to-digital converter interface, and pClamp 8.0 software (Axon Instruments, Foster City, CA). Signals were digitized at 25 kHz and low-pass filtered at 3 kHz for action potential measurement and 10 kHz sampling with 1 kHz low-pass filtering was done for slow inward current measurement. Electrodes were pulled from borosilicate glass capillaries (P-97, Sutter instruments, Novato, CA). Initial resting membrane potential (Vₜₐ₅) was measured immediately after whole-cell configuration was obtained and the recording was only continued when the Vₜₐ₅ was more negative than -45 mV. Liquid junction potentials were calculated using JPCalc in pClamp software and all membrane potential measurements were corrected during analyses. Cell capacitance, membrane resistance, and series resistance were measured in voltage-clamp configuration using the membrane test of pClamp software 5 min after whole-cell configuration was achieved. Only neurons having series resistance lower than 10 MΩ after being compensated up to 70 % were included in analysis.

**Voltage-clamp**
In voltage-clamp experiments, the pipettes resistances were 2 to 4 MΩ when filled with internal solution, consisting of (in mM) 140 KCl, 20 HEPES, 10 EGTA, 1 MgCl₂, and 2 Mg-ATP (pH = 7.3, 280 mOsmol l⁻¹). Ih was activated by stepping the voltage from a holding potential of -60 mV to between -40 mV and -120 mV for 2 seconds in -10 mV increments, followed by -100 mV for 1 second. Each current trace was digitally subtracted using Clampfit with parallel trace run in the presence of 4-((N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride (ZD7288) (Tocris, 100 μM), a specific Ih inhibitor (BoSmith et al. 1993; Harris and Constanti 1995), to acquire the Ih component only. Only neurons conducting with a slowly activating inward current after ZD7288 subtraction were used.

The voltage dependence of Ih activation at steady-state was examined by measuring the tail current at -100 mV final common step after a series of different potentials was applied to activate various degrees of Ih (Banks et al. 1993; Doan and Kunze 1999). The tail current amplitudes were normalised to the maximal tail current amplitude and plotted against the corresponding preceding voltage step. The normalised current-voltage relationship was fitted in GraphPad Prism (GraphPad Software, San Diego, CA, USA) with a Boltzmann equation in the following form:

$$\frac{I_t}{I_{t(max)}} = \frac{1}{1+\exp[(V_m-V_{1/2})/s]}$$

where $V_m$ is the membrane potential of the pre-pulse, $V_{1/2}$ is the half-activation potential, $s$ is the slope factor, $I_t$ is the current amplitude of the tail current recorded for a given pre-pulse and $I_{t(max)}$ is the maximum tail current amplitude. To determine the onset kinetics of Ih, time constants were obtained by fitting current traces (from the instantaneous peak to 2 seconds) with a sum of two exponential functions in the following form:
\[ I_h(t) = A_{\text{fast}} \exp(-t/\tau_{\text{fast}}) + A_{\text{slow}} \exp(-t/\tau_{\text{slow}}) \]

Where \( I_h(t) \) is the amplitude of the current at time \( t \), \( A_{\text{fast}} \) and \( A_{\text{slow}} \) are the amplitudes of the fast and slow components, and \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) are the activation time constant, respectively.

Current clamp

In current clamp experiments, the pipette resistances were 8 to 10 M\( \Omega \) when filled with internal solution, consisting of (in mM) 120 K-methansulfonate, 20 KCl, 5 NaCl, 10 HEPES, 10 EGTA, 1 MgCl\(_2\) and 2 Mg-ATP (\( \text{pH} = 7.3 \), 280 mOsmol l\(^{-1}\)). The stability of the resting membrane potential was checked for at least 1 min prior to evoking action potentials and was monitored throughout the recording. Then, a series of brief depolarising current steps (100 pA step, 1 ms) were injected to evaluate action potentials in a fast current-clamp mode (Magistretti et al. 1996). Action potential parameters such as the duration at 50 % of amplitude (action potential half-duration), the maximal rate of rise (\( \text{d}V/\text{d}t_{\text{max}} \)), the maximal rate of fall (\(-\text{d}V/\text{d}t_{\text{max}}\)), and the afterhyperpolarisation (AHP) 10 to 90 % recovery time (AHP repolarisation time) were measured.

The pattern and the number of action potentials discharged were determined by injecting a series of depolarising current steps (500 ms duration). The minimum current required to evoke the first action potential (rheobase) was determined by current injection steps (50 - 100 pA increments), then a current step 3 times rheobase was applied. A series of hyperpolarising current steps (25 - 50 pA increment, 500 ms duration) were also applied to check the rectification of the cell. A rectification index (%) of the voltage responses to hyperpolarising current was calculated as
[(V_{peak} - V_{steady})/ V_{peak}] \times 100 \text{ in both control and ZD7288 applied conditions (Viana et al. 2002). The rectification index increased as V_{peak} was more hyperpolarised and the maximum index value with stable recording (V_{peak} = -100 mV to -120 mV) was compared. The rectification index in the presence of ZD7288 was between 1 and 4 % therefore the neurons with a rectification index of more than 5 % were considered to have I_h (Cabanes et al. 2002).}

**Drug application**

ZD7288 was dissolved in distilled water (100 mM, stock concentration) and capsaicin (Sigma, Australia) stock solution was made in 100 % ethanol (10 mM) and stored at 4 °C. Both stock solutions were diluted to working concentrations (dilution factor = 1:1000) in HEPES buffered saline before use. Drugs were perfused by a gravity driven valve controller system (VC-6M, Warner Instruments, USA). The flow rate was 0.25 ~ 0.3 ml min^{-1} and the application tube was placed at approximately 0.5 to 1 mm to minimise the flow pressure effect on the cell and to reduce drug diffusion.

**Western blot**

Sprague-Dawley rats were deeply anaesthetised with isoflurane (4 %) and decapitated. Both trigeminal ganglia were quickly removed and sonicated immediately in ice-cold lysis buffer (CelLytic MT, Sigma) with phenylmethanesulfonyl fluoride (50 μM, Sigma) and protease inhibitor cocktail (1:100, Sigma P8340). Lysates were centrifuged and the protein concentration of the supernatant was measured using the BioRad Protein Assay (Regent's Park, Australia). Equal amounts of protein (30 μg) were separated on an 8 % sodium dodecyl sulphate-
polyacrylamide gel. The protein was then transferred overnight at 30 mV at 4 °C onto a
polyvinylidene difluoride membrane (Hybond-P, Amersham, Melbourne, Australia).
Membranes were blocked overnight at 4 °C in a blocking solution consisting of 5 % skim milk
powder in PBS-T (PBS plus 0.1 % Tween 20). Blots were then incubated with rabbit anti-HCN
antibody (dilution 1:250 for anti-HCN1, 1:2000 for anti-HCN2, 1:500 for anti-HCN3 and 1:200
for anti-HCN4) and rabbit anti α-actin antibody (dilution 1:2000, Sigma) overnight at 4 °C.
Washes were done with PBS-T (3 times, 15, 5, and 5 min each). Blots were then incubated with
an HRP-conjugated donkey anti-rabbit antiserum (dilution 1:2000, Amersham) for 1 hour at
room temperature. After a final wash, protein immunoreactivity was visualised by enhanced
chemiluminescence (ECL, Amersham) and exposed to Hyperfilm ECL (Amersham). Molecular
weight was estimated using Precision Plus Protein Standards (BioRad). The band intensity
signal was quantified using Image J software (v1.32j, National Institute of Health, USA).

**HCN antibodies**

The following antibodies were used in this study: 1) rabbit anti-HCN1 raised against 849-862
(QAGSRSTVPQRVT) of the rat HCN1 protein; 2) rabbit anti-HCN2 raised against 849-863
(CLDPLDSARSRLSSNL) of the rat HCN2 protein; 3) rabbit anti-HCN3 raised against 586-599
(RGLAPGTGARLSGKL) of the rat HCN3 protein; 4) rabbit anti-HCN4 raised against 119-155
(HGHLHDSAEERRLIAEGDASPGEDRTPPGLAAEPERP) of the human HCN4 protein and
with rat HCN4 protein sharing 35/37 residues. Anti-HCN1, anti-HCN2, and anti-HCN3 were
gifts from GlaxoSmithKline (Harlow, UK) and peptide pre-incubation tests for HCN1 and HCN2
were done previously in our lab (Cho et al. 2009a; Xiao et al. 2004). The antigen peptide that the
antibody to HCN3 was raised against was synthesised (Auspep Pty Ltd, Parkville, VIC, Australia) for a pre-incubation experiment. Pre-absorption of the anti-HCN3 primary antibody for 24 hours with antigen peptide (100 \( \mu \text{g ml}^{-1} \)) abolished the immunoreactive band completely. Anti-HCN4 was purchased from Alomone laboratory (Jerusalem, Israel) and has been previously characterised (Cho et al. 2009b; Much et al. 2003).

Statistics

All data were tested for normality (Kolmogorov-Smirnov test; GraphPad Prism) and appropriate parametric (Student t-test (paired or unpaired) or one-way ANOVA) or non-parametric (Mann-Whitney (unpaired) tests, Wilcoxon signed rank test (paired), or Kruskal – Wallis test with Dunns post test (for multiple group comparison) were used. The Spearman test for correlation of non-parametric distributions was also used. A probability of \( P < 0.05 \) was considered significant. Normally distributed data is presented as mean ± SEM; data are otherwise presented as median (25\(^{th}\) – 75\(^{th}\) percentile).
Results

In the present study, whole-cell voltage-clamp or current-clamp recordings from acutely dissociated TG neurons from rats of 1, 7, 14, 21, and 35 postnatal days (P) were used for analysis. For simplicity, data from cells at P1 and P35 are presented in this section and all data, including P7, P14, and P21, are summarised in the tables.

Passive properties of \( I_h \) positive neurons

Small- medium sized, capsaicin sensitive neurons were investigated as these sensory neurons have previously been shown to express \( I_h \) (Momin et al. 2008; Scroggs et al. 1994; Tu et al. 2004). \( I_h \) could be recorded from all neurons examined. In addition, ZD7288 (100 μM) was applied to all neurons following the initial characterisation. The resting membrane potential was more depolarised in older animals compared to younger animals, it was \(-62.1 \pm 0.8 \text{ mV} \) (\( n = 21 \)) at P1 and \(-59.4 \pm 0.9 \text{ mV} \) (\( n = 21 \)) at P35 (one-way ANOVA of P1, P7, P14, P21, and P35, \( P<0.01 \)). The cell capacitance, an indication of cell size, increased from \( 24.0 \pm 2.0 \text{ pF} \) (\( n = 21 \), P1) to \( 52.6 \pm 5.0 \text{ pF} \) (\( n = 21 \), P35, ANOVA, \( P<0.0001 \)) and the membrane resistance significantly decreased with postnatal maturity, from a median of \( 266 \text{ MΩ} \) (25\textsuperscript{th} percentile, 212 \text{ MΩ} – 75\textsuperscript{th} percentile, 395 \text{ MΩ}, \( n = 21 \), P1) to \( 148 \text{ MΩ} \) (92 – 205 \text{ MΩ}, \( n = 21 \), P35; Kruskal-Wallis test, \( P<0.05 \)). Consistent with the decrease in membrane resistance, the minimum current to evoke the first action potential (rheobase) increased from \( 382 \pm 44 \text{ pA} \) (P1, \( n = 11 \)) to \( 722 \pm 239 \text{ pA} \) (P35, \( n = 10 \)) but was not significant (ANOVA, \( P>0.05 \), Table 1).
Properties of Ih in developing TG neurons

In current-clamp experiments, Ih was identified by the presence of a depolarising voltage sag following hyperpolarising current steps (Fig 1 A, top). The current–voltage (I-V) relationship of peak and steady-state potentials of P1 and P35 is shown in Fig 1 B. In neonatal neurons, the peak hyperpolarised voltage was greater than that of adults indicating a higher membrane resistance (Table 1). The changes in voltage response from peak hyperpolarisation (i.e. depolarising sag, between -100 mV and -120 mV) to steady state was calculated and presented as a rectification index (see (Cabanes et al. 2002; Viana et al. 2002)). The rectification index was not different between adults and neonates; 24.5 ± 2.1 % (P1, n = 11) and 19.7 ± 2.8 % (P35, n = 10, ANOVA, P>0.05; Table 1). A few minutes after ZD7288 application, the depolarising sag was abolished and less current was required to cause hyperpolarisation indicating that input resistance was increased (Fig 1 A, middle).

The slowly developing, non-inactivating inward current, Ih, recorded in voltage clamp made, was activated by stepping the membrane voltage from a holding potential (-60 mV) in a series of voltage steps (-40 mV to -120 mV). Membrane voltages more negative than -120 mV usually resulted in unstable current traces at 35 °C, but at room temperature (RT) hyperpolarising the membrane to -130 mV produced a stable current trace. However, at RT, Ih often did not reach a steady-state, even after 4 seconds while it reached the steady-state within 2 seconds in most cases at 35 °C. These observations are consistent with the previous findings that Ih is highly temperature dependent (Q₁₀ = 3 to 5 (Hogg et al. 2001; Magee 1998; Orio et al. 2009; Pape 1996) Fig 2 D, E, F).
This slow inward current completely disappeared following ZD7288 application in a time dependent manner (the full effect required at least 1 min exposure to ZD7288, Fig 2 B). Control experiments were carried out where run down was recorded. The ZD7288 effect was within 2-3 min of application and completely abolished $I_h$ while rundown is not obvious during this short period of time and was usually only a small reduction of $I_h$. The remaining current is thought to comprise of an inward rectifying potassium current and a leak current (Scroggs et al. 1994). $I_h$ density was calculated from the amplitude of ZD7288 specific current (Fig 2 C) divided by cell capacitance (an indirect measure of membrane surface area). Although $I_h$ density was highly variable, the maximum current density measured at -120 mV was not significantly different between age groups, such that the median was -15.8 pA pF$^{-1}$ (-3.7 to -26.8 pA pF$^{-1}$) at P1 ($n = 9$) and -8.7 pA pF$^{-1}$ (-6.5 to -18.8 pA pF$^{-1}$) at P35 ($n = 11$, Kruskal-Wallis test, $P > 0.05$, Fig 2 E, F, Table 2). While the median $I_h$ density decreased during maturation, there was no significant correlation between age and density (Spearman test, $P > 0.05$). In the studies done at RT, the maximum current amplitude was measured 4 seconds after a -130 mV hyperpolarising voltage step. The $I_h$ density, however, was smaller than that measured at -120 mV at 35°C (significant at P7, -8.6 ± 1.3 pA pF$^{-1}$, $n = 14$ compared to -15.4 (-10.5 to -35.9 pA pF$^{-1}$), $n = 13$, Mann-Whitney, $P < 0.01$). At RT, there was also no difference in $I_h$ density (Kruskal-Wallis test, $P > 0.05$) during postnatal maturation (Fig 2F, Table 2).

The voltage dependence of $I_h$ activation was presented as an activation curve derived from tail current normalisation (Fig 2 G). $I_h$ activation was observed from around -50 mV and reached a
maximum at -120 mV (-130 mV at RT), the most hyperpolarised potentials used in this study. The activation curve shifted to more depolarised potentials with postnatal age such that the half maximal activation potential ($V_{1/2}$) moved from $-89.4 \pm 2.3$ mV (n = 9, P1) to $-81.6 \pm 1.4$ mV (n = 11, P35, ANOVA, $P<0.001$) without changing the slope factor (7.8 ± 1.0 at P1 and 8.3 ± 0.7 at P35, ANOVA, $P>0.05$). At RT, the $V_{1/2}$ was more hyperpolarised compared to that recorded at 35°C (e.g. $-97.8 \pm 2.4$ mV, n = 15, P1, Student t-test, $P>0.05$). At RT, the $V_{1/2}$ also became significantly more depolarised with postnatal age ($-85.8 \pm 2.9$ mV at P35, n = 8, ANOVA, $P<0.001$, Fig 2 G, H and Table 2).

To determine the reversal potential of $I_h$ the linear instantaneous current – voltage relationship ($I_{inst} - V$) at voltage steps (-60 mV to -100 mV, -10mV increment) from a holding potential, -60 mV or -100 mV was obtained to get the chord conductance at each holding potential. Extrapolation of the two lines yields a reversal potential of $I_h$ where the conductance difference becomes zero. The reversal potential of $I_h$ estimated with this method was $-37.1 \pm 3.0$ mV in TG neurons (n = 14 from animals 14 to 35 days old, there was no significant age-dependent difference).

**HCN protein subtype changes in developing TG**

We also tested for the presence of all subtypes of HCN protein at different postnatal ages (P1, P7, P14, P21 and P35) in rat TG using Western blot (Fig 3). Cortical brain tissue from a P21 rat was used in every experiment as a positive tissue control (Notomi and Shigemoto 2004). Bands
immunoreactive for each of the HCN subtypes were detected in both rat trigeminal ganglia and brain around the corresponding molecular weight for rat HCN1 (102 kDa), rat HCN2 (95 kDa), rat HCN3 (87 kDa), and rat HCN4 (129 kDa) (Fig 3 A). The optical density (OD) of each HCN band was normalised against the α-actin band density, which was used as a loading control (Fig 3 B). The HCN1 band density increased most quickly to P14 and then reached a plateau such that the HCN1 band at P1 was visible (0.47 ± 0.10 OD, n = 6) although lighter than the mature levels at P14 (0.93 ± 0.15 OD, n = 6, Student’s t-test, P<0.05 compared to P1). The HCN2 band density was hardly detected at P1 (0.19 ± 0.03 OD, n = 6) but continuously increased until P35 (1.27 ± 0.26 OD, n = 5, Student’s t-test, P<0.05 compared to P1). HCN3 was very faint and did not significantly change with postnatal maturation (0.14 ± 0.08 OD at P1 and 0.28 ± 0.05 OD at P35, n = 5 each, Student’s t-test, P>0.05 compared to P1). The HCN4 protein band was visible at P1 (0.59 ± 0.13 OD, n = 4) and did not change significantly over the 5-week time period examined (0.84 ± 0.22 OD, n = 4, Student’s t-test, P>0.05 compared to P1). Comparison of the relative ratio of each subtype revealed that the ratio of HCN4 showed a clearly decreasing pattern until P14 after which it remained stable (Fig 3 C).

As all HCN subtypes were detected in trigeminal ganglia in the current study (Fig 3) and each subtype has been shown to have different activation kinetics when expressed heterologously (Moosmang et al. 2001; Stieber et al. 2005), the onset kinetics of $I_h$ were analysed and compared with the protein level changes during development. $I_h$ evoked by hyperpolarising voltage steps was well fitted with a sum of two exponential functions and two time constants, $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$, were derived (Fig 4 A). Both the time constants were voltage dependent and were quicker at more hyperpolarised holding potentials. They were also age dependent such that at the -90 mV
voltage step, both $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ were significantly faster in older animals (65.1 ± 7.6 ms and 490.2 ± 68.3 ms, n = 11, P35) than in younger animals (198.8 ± 42.9 ms and 980.6 ± 95.7 ms, n = 10, P1, ANOVA, $P<0.01$ in both $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$). There was no significant change in the amplitude ratio of fast and slow component [$A_{\text{fast}}/(A_{\text{fast}} + A_{\text{slow}})$] between ages (ANOVA, $P>0.05$, Fig 4 B, C and Table 2).

In experiments at RT, current activation time constants were significantly slower than those at 35 °C. For example, at P1, $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ were 425.0 ± 39.9 ms and 2603.7 ± 418.2 ms, respectively (Student t-test, $P<0.001$ and $P<0.01$, respectively, Fig 4 B, C and Table 2). As in the experiments at 35 °C, both time constants were also faster with postnatal maturation. Although the time constants decreased and cell capacitance increased with age (seen as negative correlation in Fig 4 D, E), the correlation between the time constant and cell capacitance at an individual age group was not obvious and was only significant for $\tau_{\text{fast}}$ at P1 (RT) and $\tau_{\text{slow}}$ at P7 (35 °C, Fig 4 D, E).

**Role of $I_h$ in neuronal excitability**

The resting membrane potential measured 5 min after achieving whole-cell configuration was depolarised in older rats (-60.5 ± 0.8 mV, n = 10, P35) compared to neonates (-65.1 ± 1.3 mV, n = 11, P1, ANOVA, $P<0.001$). As $I_h$ activation can be observed from around -50 mV in postnatal sensory neurons (Fig 2 G), we hypothesised that blocking $I_h$ (a depolarising current) would hyperpolarise the resting membrane potential. Within 3 min of ZD7288 application, the
membrane potential of cells of all ages examined was significantly hyperpolarised in a time
dependent manner, for example, at P1, from -65.1 ± 1.3 mV to -67.5 ± 1.4 mV (n = 11, Student’s
t-test, P<0.05, Fig 5, Table 3).

To investigate how $I_h$ activation affected the active membrane properties of developing TG
nociceptive neurons, the shapes of single action potentials evoked by a short depolarising pulse
(1 ms) before and during application of ZD7288 were compared. Fig 5 A shows an example of
an action potential in a TG neuron with a spike followed by an AHP. ZD7288 increased the
action potential half-duration (from 0.5 ± 0.1 ms to 0.8 ± 0.1 ms at P1 and from 0.7 ± 0.1 to 1.1 ±
0.1 ms at P35, Student’s t-test, $P<0.001$ in both groups) and slowed the maximal rate of rise and
fall of action potentials significantly at P1, P14, and P35. The rise rate decreased from 240.6 ±
29.8 to 187.1 ± 23.3 mV ms$^{-1}$ at P1 (Student’s t-test, $P<0.01$) and 201.3 ± 18.3 to 138.4 ± 8.1 mV
ms$^{-1}$ at P35 (Student’s t-test, $P<0.01$, Fig 5 B). The falling rate also decreased from -107.4 ±
15.1 to -88.6 ± 9.3 mV ms$^{-1}$ at P1 (Student t-test, $P<0.05$) and from -93.5 ± 5.2 to -70.4 ± 4.2 mV
ms$^{-1}$ at P35 (Student t-test, $P<0.01$, Fig 5 B). The 10 - 90% decay time of the AHP (from its
peak, back to the resting membrane potential) also increased from 0.19 ± 0.03 to 0.29 ± 0.05 ms
at P1 and from 0.24 ± 0.02 to 0.43 ± 0.04 ms at P35 (Student’s t-test, $P<0.05$ at P1, $P<0.01$ at
P35, Fig 5 A). Parameters at all postnatal time points are summarised in Table 3.

As previous studies in adult sensory neurons have revealed an important role of $I_h$ in generating
repetitive discharge (Hogan and Poroli 2008; Momin et al. 2008), we investigated the
involvement of $I_h$ in repetitive firing of developing TG neurons by applying a depolarising
current step (500 ms, 3 times rheobase). Most neurons studied at P1 (10/11) showed only a
single spike even at 3 times rheobase current injection while multiple firing neurons were frequently observed in older animals (8/10 at P35, Fig 6). After ZD7288 application, the firing frequency of the multiple firing neurons at P35 was significantly reduced from a median of 26 Hz (15 – 95 Hz) to 10 Hz (4 – 49 Hz, Wilcoxon matched pairs test, P<0.05, Fig 6 B).

At the end of the above current-clamp recordings, the amplifier mode was switched to voltage-clamp to examine capsaicin (3 µM) responses, an agonist of transient receptor potential vaniloid 1 (TRPV1).
**Discussion**

The present study shows that HCN channel protein and $I_h$ conducted through the channel are present from birth and are able to influence the excitability of sensory neurons at all postnatal ages. The present observation that $I_h$ both becomes active at more depolarised potentials and has faster activation kinetics suggests that $I_h$ contribution to somatosensory and nociceptive processing in sensory neurons is also likely to increase during development.

$I_h$ is activated in developing TG neurons

$I_h$ was found in TG neurons of rats from newborn to 5 week old (the oldest investigated). Other studies have reported $I_h$ in adult rat DRG and TG neurons, where it occurs in 100 %, 92 %, and 45 % of large, medium, and small-sized neurons, respectively (Cabanes et al. 2002; Momin et al. 2008; Scroggs et al. 1994; Tu et al. 2004). In the current study, small to medium-sized capsaicin sensitive neurons were preferentially recorded at every age and the properties of $I_h$ were compared. Our data, cell diameter (17.8 ± 0.5 μm at P1 and 25.3 ± 0.9 μm at P35) and membrane capacitance (52.6 ± 5.0 pF at P35) falls within the parameter described by previous studies for small to medium-sized cells (Cabanes et al. 2002; Scroggs et al. 1994).

Few other studies have examined $I_h$ development in sensory neurons. In one, $I_h$ was first detected around embryonic day 10 in both TG and DRG of quail and was present in 75 % of neurons on the first postnatal day (Schlichter et al. 1991). In another (published in abstract form only), $I_h$ is present in 20 % of rat DRG neurons at P1, increasing to 100 % at P14 (Fulton 1987). All of these studies were done at room temperatures. In the current study, a few small sized
neurons with no $I_h$ were found at each postnatal age in recordings at room temperature, however, all cells sampled at 35 °C exhibited an $I_h$.

The density of $I_h$ (the amplitude of $I_h$ per unit of cell capacitance) showed a great degree of variability at 35 °C, but was not significantly different in neurons of any postnatal age. The variability of current density has been previously reported in embryonic (Schlichter et al. 1991) and adult sensory neurons, even within cells of a similar size, (Petruska et al. 2000; Scroggs et al. 1994) reflecting the heterogeneous nature of sensory neurons. Similarly, there was little change during postnatal development in the degree of rectification caused by $I_h$ (Table 1). This has previously been reported in mouse TG neurons during early postnatal development (Cabanes et al. 2002). Since neither $I_h$ density nor rectification index changes significantly over the postnatal period, it is likely that $I_h$ is important in neonatal sensory neuronal function from birth to adult. However, activation became more rapid and the activation voltage curve was shifted to more depolarised voltages between P1 and P35, suggesting that $I_h$ has a greater role in shaping neuronal responses as rats mature (see below).

**HCN subtype proteins and $I_h$ properties change in developing postnatal TG neurons**

The relative levels of the HCN channel proteins were developmentally regulated. All HCN channel protein subtypes were detected by Western blot at every postnatal age, with levels of HCN1 and HCN2 proteins increasing and the proportions of HCN4 protein decreasing with age (Fig 3). The relative abundance of HCN protein is associated with differences in the biophysical
properties of \( I_h \), e.g. kinetics, voltage dependence, and cAMP sensitivity (Kanyshkova et al. 2009; Santoro et al. 2000; Surges et al. 2006). The increasing levels of HCN1 and HCN2 proteins and/or the decreasing proportions of HCN4 protein, the slowest activating channel, during postnatal maturation might contribute to the accelerated channel activation kinetics seen in older neurons (Fig 4). In addition, significant increases of HCN1 and HCN2 proteins are likely to contribute to the \( V_{1/2} \) shift to more depolarised potentials (Fig 2 E) since HCN1 has the most depolarised \( V_{1/2} \) in basal conditions (Stieber et al. 2005).

Despite the greater amount of protein that was revealed by Western blot analysis, \( I_h \) current density remained the same during development. This implies that the proportion of protein in the ganglion that is functional, in that it contributes to membrane current, decreases during development. This could mean that there is more protein in reserve that could be inserted into the membrane and contribute to plastic changes in the neurons. A proportion of protein that is non-functional in the nerve cells could be destined for export to the nerve terminals.

Experiments in heterologous expression systems have shown that both homomeric and heteromeric HCN channels can form (Much et al. 2003). In individual DRG neurons, there is immunohistochemical evidence for more than one HCN channel subtype, suggesting the presence of heteromeric channels (Chaplan et al. 2003; Tu et al. 2004). Co-localisation was not investigated in individual TG neurons in the three studies published to date (Cho et al. 2009a; Cho et al. 2009b; Wells et al. 2007). However, we have shown that HCN1 and HCN2 are found in similar sized neurons (Cho et al. 2009a). A previous study reported that the \( I_h \) activation kinetics were faster in large-sized than in small-sized DRG neurons recorded at RT (Momin et al.
2008). In the current study this relationship between cell-size and activation kinetics was only apparent in studies at RT at P1 and in studies at 35 °C at P7. However, our sample did not cover the entire range of sensory neuron sizes and there were developmental changes in the relative distribution of HCN channel subtypes and significant increases in activation kinetics with age.

\textit{I}_h \textit{is involved in neuronal excitability of developing TG neurons}

The best characterised role of \textit{I}_h is as in rhythm generation in cardiac cells (DiFrancesco 1993) and in other spontaneous firing neurons (Maccaferri and McBain 1996; McCormick and Pape 1990). In some excitable neurons, \textit{I}_h contributes more to determining passive membrane properties such as a resting membrane potential and input resistance (Williams et al. 2002). In these cells \textit{I}_h tends to be a membrane stabilizing current, increasing at hyperpolarised potentials and decreasing with depolarisation.

In the present study, \textit{I}_h was active at rest, and it enhanced repetitive action potential firing, as shown by the action of ZD7288, which hyperpolarised the neurons by 2 mV and reduced the frequency of action potentials elicited by a current at 3 times rheobase from 26 to 10 Hz. Some studies reported that ZD7288 increased the frequency of AP as a result of increased input resistance (Doan et al. 2004; Li et al. 2008). In our study, input resistance was slightly increased following ZD7288 but that was not enough to increase the excitability. This discrepancy could be due to the different cell types that were studied. Both papers that showed increased
excitability, used nodose ganglion neurons while all studies in sensory neurons, DRG and TG neurons, have found decreased excitability.

In response to prolonged intracellular injection of depolarising current, mature TG neurons either discharge repetitively or fire only single action potentials (Catacuzzeno et al. 2008). We found that most P1 to P7 TG neurons fired a single action potential and observed multiple firing neurons more frequently at later developmental stages. This increase in the firing frequency of DRG neurons at older postnatal ages has been observed in vivo (Fitzgerald 1987) and is likely to reflect the developmental regulation of voltage-gated ion channels, including Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\) channels (Fedulova et al. 1991; Rush et al. 2007; Seifert et al. 1999). The results of the present work suggest that HCN channels also contribute to these postnatal changes in sensory neuron firing frequency.

Roles of \(I_{h}\) in shaping the action potential and in determining firing properties

ZD7288 also changed the action potential shape, which had a longer half-duration, slower rate of rise and fall, and a prolonged recovery of the AHP (Fig 5, Table 3). These findings are in agreement with previous studies (Hogan and Poroli 2008; Kouranova et al. 2008; Masuda et al. 2006; Orio et al. 2009; Tanaka et al. 2003). Slower action potential rising and falling rates and longer half durations were observed following ZD7288 by Tanaka et al. (Tanaka et al. 2003) and in the present work. Because \(I_{h}\) kinetics are considerably slower than the action potential, the sculpting of the action potential is probably due to persistent \(I_{h}\) activity. During rapid depolarisation (negative to the reversal potential of the HCN channel, -37.1 mV, see results), an
inward conductance through the open HCN channels may contribute to depolarisation. On the other hand, when the membrane potential is positive to the HCN channel reversal potential during the action potential peak, the outward conductance could speed action potential repolarisation. A longer half width was also shown when I_h conductance was set at zero in a DRG neuron computational model (Kouranova et al. 2008).

During the AHP, the HCN channel is re-activated and the inward I_h depolarises the membrane until counter-balanced by outward potassium current (Pape 1996). Blocking I_h slowed the AHP decay time in TG neurons (current study) and DRG neurons (Hogan and Poroli 2008; Masuda et al. 2006). The slower AHP decay time in the presence of ZD7288 indicated that other currents contribute to the AHP. These may include a conductance through Ca^{2+} activated potassium channels (Sarantopoulos et al. 2007). This, however, needs to be clarified as ZD7288 has been found to partially block T-type voltage-gated calcium channels in some studies (Felix et al. 2003; Sanchez-Alonso et al. 2008).

**Physiological relevance**

In the present study, we showed that I_h is important in determining the excitability of sensory neurons in newborn as well as adult rats. Since all neurons responded to capsaicin, an agonist at TRPV1 receptor (predominantly expressed in nociceptive afferents) (Caterina et al. 2000); it is likely that I_h might modify nociception during postnatal maturation. In adult rats, I_h has been shown to contribute to peripheral nerve hyperexcitability (Chaplan et al. 2003; Lee et al. 2005).
In adult DRG and TG neurons, nerve injury (Chaplan et al. 2003; Kitagawa et al. 2006; Yao et al. 2003) as well as inflammation (Ingram and Williams 1996; Momin et al. 2008) result in increased \( I_h \) conductance, accelerated current activation, and positively shifted \( I_h \) activation voltages; thus contributing to the hyper-excitability of neurons in these pain states. The present observation that \( I_h \) both becomes active at more depolarised potentials and has faster activation kinetics suggests that \( I_h \) contribution to somatosensory and nociceptive processing in sensory neurons is also likely to increase during postnatal development. This may contribute to the poor following characteristics of neonatal rat sensory neurons in the first postnatal week (Fitzgerald 1987).

It should be noted that the majority of \textit{in vitro} studies that have examined \( I_h \) in somatosensory neurons have been done at room temperature (Cabanes et al. 2002; Chaplan et al. 2003; Kouranova et al. 2008; Momin et al. 2008; Petruska et al. 2000; Schlichter et al. 1991; Scroggs et al. 1994; Yao et al. 2003). Results from the current study show that at room temperature \( I_h \) has slower onset kinetics and more hyperpolarised half-activation voltages when compared to recordings at 35 °C, and probably contribute less to neuron excitability as a result. In addition, a greater percentage of the population showed \( I_h \) at 35°C than at room temperature. This temperature relationship of \( I_h \) has previously been reported in TG neurons (Cabanes et al. 2003) and intracardiac neurons (Hogg et al. 2001).

\textbf{Conclusions:}
In this study, we have identified that neonatal TG neuron expresses $I_h$ with similar density to adult neurons but with different voltage dependence and kinetics. The activation curve shifted to the right with age (Fig 2G) and activation was more rapid (Fig. 5), yielding a greater prominence of $I_h$ with postnatal maturation. Thus $I_h$ has a role in trigeminal primary afferent neurons at birth, and its role increases as rats mature.

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Author contribution:

1. Conception and design of the experiments
   H-jC and EAJ designed these experiments

2. Collection, analysis and interpretation of data
   Data was collected and analysed by H-jC. EAJ and JBF also contributed to analysis, all authors contributed to interpretation.

3. Drafting the article or revising it critically for important intellectual content
   H-jC prepared the first draft of the article. H-jC, JBF and EAJ revised the article critically for intellectual content.


**Figure legends**

**Fig 1 Membrane recordings of \( I_h \) in current clamp mode**

A. Top, a voltage sag was observed during hyperpolarising current injection (current step shown below traces). No voltage sag was apparent following ZD7288 application (middle trace). Note the larger voltage change in response to the same current injection due to increased membrane resistance. B. Mean (± SEM) peak voltage change (circles) and steady-state voltage change (ss, squares) plotted against hyperpolarising current for trigeminal sensory neurons at P1 and P35.

**Fig 2 Age-related characteristics of \( I_h \) revealed in voltage clamp experiments**

A. The time-course of the onset of the inward current activated by hyperpolarising voltage steps in a voltage-clamp experiment from a P35 rat TG neuron. B. No slowly activating inward current was apparent when the same voltage step protocol was run in the presence of ZD7288 (100 μM). C. The residual current (A-B). Insets to the left are the early phases of current activation on an expanded time scale. D. Example of \( I_h \), activated by a -90 mV hyperpolarising voltage step, which did not reach a steady-state, even at 4 seconds when recorded at room temperature (RT) but was almost fully activated by 2 seconds at 35 °C. The current traces were normalised to the maximum current amplitude of each trace for better comparison of the time course. E. Current density [(Steady state current amplitude (square) - instantaneous current amplitude (circle) / cell capacitance)] of \( I_h \) activated by hyperpolarising voltage steps. The error bars have been omitted for clarity and these values are shown in Table 2. F. The maximum \( I_h \) density at -120 mV (35 °C) and at -130 mV (RT) at different postnatal ages. For panels F and H, asterisk indicates the significance of data when compare to P1 and hash symbol indicates is the
significance when compared within the same age group to recordings at 35 °C. (* or #: P<0.05, ** or ##: P<0.01, *** or ###: P<0.001). G. Normalised I_h voltage activation curves, derived from tail currents. H. I_h V_{1/2} at different ages. V_{1/2} was significantly more depolarised at older postnatal ages.

**Fig 3 Western blot of HCN subtypes in TG at different postnatal ages**

A. Immunoreactive bands of all four isoforms were detected with different densities dependent on age. In the right lane, P21 cortical brain tissue was used in every experiment as a positive tissue control. Each HCN band density was normalised to the α-actin band density examined from the same membrane. B. The normalised band density of HCN subtypes at different postnatal ages (labelled as gray to black as indicated at the top). Asterisk indicates the significance when compared to P1. C. The ratio of individual HCN subtypes (as a percentage of total HCN) expression levels at different postnatal ages.

**Fig 4 Onset kinetics of I_h**

A. Example of I_h activated by -90 mV voltage step in trigeminal ganglion neurons aged P1 (left) and P35 (right). Activated current traces were fitted with the sum of two exponential functions (grey line), each of which is shown as a dashed line. The current traces were normalised to the maximum current amplitude of each trace for better comparison of the time course. B, C. The time constants of fast (B) and slow component (C) are plotted against activated voltage (left) and postnatal age (right). Asterisks indicate the significance of data when compare to P1 and hash symbols indicate the significance when compared with the same age group to recordings at 35 °C. (* or #: P<0.05, ** or ##: P<0.01, *** or ###: P<0.001). D, E The fast (D) and slow (E)
time constants at -90 mV plotted against the cell capacitance from 35°C (left) and RT recordings (right).

**Fig 5 Shape of action potential before and after ZD7288**

A. Single action potential (AP) evoked by brief (1 ms) depolarising current injection shown in black (control) and grey (after ZD7288). B. 1st derivative (dVm dt⁻¹) of the voltage trace shown above. Bottom, current injected. Parameters measured: a, resting membrane potential; b, AP duration at 50 % of amplitude; c, AP maximum rate of rise; d, AP maximum rate of fall; e, afterhyperpolarisation (AHP) decay time between 10 to 90 % of amplitude.

**Fig 6 Effect of ZD7288 in the repetitive firing of TG neurons**

A. Repetitive action potentials were evoked by (500 ms) depolarising (3 times rheobase) current injections (bottom). Examples of voltage responses in control conditions (black) and after ZD7288 application (grey) at P1 (top) and P35 (middle). B. The AP frequency was significantly decreased after ZD7288 application at P35, but not at other ages. The median firing frequency (horizontal bar) with 25th, 75th percentile (error bar) is plotted, for the multiple firing neuron population.

Tables:
Table 1 Summary of passive membrane properties and rectification of sampled TG neurons

<table>
<thead>
<tr>
<th></th>
<th>V_m (mV)</th>
<th>n</th>
<th>C_m (pF)</th>
<th>n</th>
<th>R_m (MΩ)</th>
<th>n</th>
<th>R_b (pA)</th>
<th>n</th>
<th>R_index (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>-62.1 ± 0.8</td>
<td>21</td>
<td>24.0 ± 2.0</td>
<td>21</td>
<td>266 (212 - 395)</td>
<td>21</td>
<td>382 ± 44</td>
<td>11</td>
<td>24.5 ± 2.1</td>
<td>11</td>
</tr>
<tr>
<td>P7</td>
<td>-60.0 ± 1.2</td>
<td>19</td>
<td>36.0 ± 2.4</td>
<td>19</td>
<td>181 (117 - 331)</td>
<td>19</td>
<td>312 ± 71</td>
<td>6</td>
<td>20.6 ± 3.0</td>
<td>6</td>
</tr>
<tr>
<td>P14</td>
<td>-58.8 ± 1.0</td>
<td>26</td>
<td>42.8 ± 3.4</td>
<td>26</td>
<td>170 (81 - 310)</td>
<td>26</td>
<td>422 ± 120</td>
<td>11</td>
<td>25.5 ± 1.8</td>
<td>11</td>
</tr>
<tr>
<td>P21</td>
<td>-55.7 ± 1.0</td>
<td>14</td>
<td>47.5 ± 2.9</td>
<td>14</td>
<td>152 (91 - 203)</td>
<td>14</td>
<td>NE</td>
<td></td>
<td>NE</td>
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<tr>
<td>P35</td>
<td>-59.4 ± 0.9</td>
<td>21</td>
<td>52.6 ± 5.0</td>
<td>21</td>
<td>148 (92 - 205)</td>
<td>21</td>
<td>722 ± 239</td>
<td>10</td>
<td>19.7 ± 2.8</td>
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<tr>
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<td>&lt;0.0001</td>
<td></td>
<td>&lt;0.05</td>
<td></td>
<td>&gt;0.05</td>
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Mean ± SEM or Median (25th - 75th percentile) are given for each parameter. V_m, resting membrane potential; C_m, membrane capacitance; R_m, membrane resistance; R_b, rheobase; R_index, rectification index; NE, no experimental data; NS, not significant.

Table 2 Summary of I_h properties of sampled TG neurons

<table>
<thead>
<tr>
<th></th>
<th>I_h density (pA pF⁻¹)</th>
<th>n</th>
<th>V_1/2 (mV)</th>
<th>Slope factor</th>
<th>Tau fast (ms)</th>
<th>Tau slow (ms)</th>
<th>A_fast/(A_fast+A_slow)</th>
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<td>35°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>-15.8 (-3.7 - -26.8)</td>
<td>9</td>
<td>-89.4 ± 2.3</td>
<td>7.8 ± 1.0</td>
<td>198.8 ± 42.9</td>
<td>980.6 ± 95.7</td>
<td>0.45 ± 0.06</td>
<td>10</td>
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<tr>
<td>P7</td>
<td>-15.4 (-10.5 - -25.9)</td>
<td>13</td>
<td>-85.8 ± 0.6</td>
<td>9.0 ± 0.5</td>
<td>124.3 ± 24.3</td>
<td>923.8 ± 155.6</td>
<td>0.55 ± 0.06</td>
<td>13</td>
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<td>P14</td>
<td>-12.0 (-5.9 - -27.4)</td>
<td>15</td>
<td>-85.6 ± 1.0</td>
<td>8.2 ± 0.8</td>
<td>104.2 ± 14.5</td>
<td>863.3 ± 99.3</td>
<td>0.51 ± 0.06</td>
<td>15</td>
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<td>P21</td>
<td>-8.9 (-4.2 - -19.4)</td>
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<td>-82.6 ± 0.9</td>
<td>8.9 ± 1.0</td>
<td>94.7 ± 12.3</td>
<td>616.7 ± 38.3</td>
<td>0.53 ± 0.05</td>
<td>15</td>
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<tr>
<td>P35</td>
<td>-8.7 (-6.5 - -18.8)</td>
<td>11</td>
<td>-81.6 ± 1.4</td>
<td>8.3 ± 0.7</td>
<td>65.1 ± 7.6</td>
<td>490.2 ± 68.3</td>
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<td>Overall</td>
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<td>&lt;0.0001</td>
<td>&lt;0.05</td>
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<table>
<thead>
<tr>
<th></th>
<th>I_h density (pA pF⁻¹)</th>
<th>n</th>
<th>V_1/2 (mV)</th>
<th>Slope factor</th>
<th>Tau fast (ms)</th>
<th>Tau slow (ms)</th>
<th>A_fast/(A_fast+A_slow)</th>
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<td>RT</td>
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<tr>
<td>P1</td>
<td>-11.3 ± 1.8</td>
<td>12</td>
<td>-98.7 ± 2.4</td>
<td>7.3 ± 0.5</td>
<td>425.0 ± 39.9</td>
<td>2603.7 ± 418.2</td>
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<tr>
<td>P7</td>
<td>-8.6 ± 1.3</td>
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<td>-99.1 ± 1.6</td>
<td>9.0 ± 0.5</td>
<td>401.0 ± 44.9</td>
<td>2469.4 ± 305.0</td>
<td>0.25 ± 0.05</td>
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<tr>
<td>P14</td>
<td>NE</td>
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<td>NE</td>
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<td>NE</td>
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<tr>
<td>P21</td>
<td>-9.5 ± 2.5</td>
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<td>-91.3 ± 1.1</td>
<td>7.9 ± 0.3</td>
<td>286.0 ± 47.3</td>
<td>1717.4 ± 211.5</td>
<td>0.45 ± 0.05</td>
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<td>-6.7 ± 1.3</td>
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<td>-85.8 ± 2.9</td>
<td>8.1 ± 0.6</td>
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Mean ± SEM or Median (25th - 75th percentile) are given for each parameter. NE, not examined; NS, not significant. I_h density was measured at -120 mV (35°C) and at -130 mV (RT).

Table 3 Summary of parameters measured as shown in Fig 5

<table>
<thead>
<tr>
<th></th>
<th>V_rest (mV, a)</th>
<th>AP halfwidth (ms, b)</th>
<th>dV/dt max (mV ms⁻¹, c)</th>
<th>-dV/dt max (mV ms⁻¹, d)</th>
<th>AHP depol time (ms, e)</th>
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<tr>
<td>P1</td>
<td>control</td>
<td>-65.1 ± 1.3</td>
<td>0.5 ± 0.1</td>
<td>240.6 ± 29.8</td>
<td>-107.4 ± 15.1</td>
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<tr>
<td></td>
<td>ZD7288</td>
<td>-67.5 ± 1.4*</td>
<td>0.8 ± 0.1***</td>
<td>187.1 ± 23.3**</td>
<td>-88.6 ± 9.3*</td>
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<tr>
<td>P7</td>
<td>control</td>
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<td>0.6 ± 0.1</td>
<td>260.1 ± 25.6</td>
<td>-106.9 ± 12.5</td>
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<tr>
<td></td>
<td>ZD7288</td>
<td>-65.4 ± 2.1</td>
<td>0.8 ± 0.1*</td>
<td>220.6 ± 22.7</td>
<td>-109.2 ± 9.4</td>
</tr>
<tr>
<td>P14</td>
<td>control</td>
<td>-62.5 ± 1.0</td>
<td>0.7 ± 0.1</td>
<td>223.1 ± 12.5</td>
<td>-108.6 ± 12.7</td>
</tr>
<tr>
<td></td>
<td>ZD7288</td>
<td>-64.1 ± 1.0</td>
<td>1.1 ± 0.1***</td>
<td>170.7 ± 14.3***</td>
<td>-85.1 ± 9.2*</td>
</tr>
<tr>
<td>P35</td>
<td>control</td>
<td>-60.5 ± 0.8</td>
<td>0.7 ± 0.1</td>
<td>201.3 ± 18.3</td>
<td>-93.5 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>ZD7288</td>
<td>-62.6 ± 1.5*</td>
<td>1.1 ± 0.1***</td>
<td>138.4 ± 8.1**</td>
<td>-70.4 ± 4.2***</td>
</tr>
</tbody>
</table>
Mean ± SEM are given for each parameter. *, ** and *** represent the significance between control and ZD7288 treated at each aged group when the P value was <0.05, <0.01 and <0.001, respectively. n = 6–11 in each group.
A

Control

ZD7288

Voltage (mV) vs Current (pA)

-140
-120
-100
-80
-60
-50
-30
-10
0
10 mV

Current injected

500 ms

100 pA

B

Current (pA)

-200
-150
-100
-50
0

Voltage (mV)

-140
-120
-100
-80
-60

peak (P1)
ss (P1)
peak (P35)
ss (P35)
A

0 mV

20 mV

control

ZD7288

c

d

b

e


B

20 V/s

200 pA

1 ms
A

P1
-63 mV

P35
-58 mV

control

ZD7288
-73 mV

10 mV

500 ms

3X reobase

B

Frequency of AP (Hz) at 3X reobase

Control
ZD7288

P1 P7 P14 P35

-63 mV -73 mV

-58 mV

-61 mV

500 ms

3X reobase